Vol. V. *August, 1903.* *No. 3*

BIOLOGICAL BULLETIN.

ON THE CONDITIONS GOVERNING THE PRODUCTION OF ARTIFICIAL PARTHENOGENESIS IN ARBACIA.

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In a previous paper[1] it was shown that sea-water concentrated by evaporation to a definite volume would produce parthenogenesis in eggs of the sea-urchin, *Arbacia*, subjected to its influence for a given length of time. During the continuation of these experiments for the purpose, primarily, of observing the morphological phenomena there has become evident a series of conditions necessary to a high ratio of parthenogenetic development. These conditions are briefly — purity of solutions, stage of development of ovarian eggs when placed in the concentrated sea-water, length of time the eggs are to remain in this solution, temperature of solutions. This article is based upon observations on eighty-three experiments, fourteen of them performed between July 25 and August 14, 1901, and sixty-nine between July 4 and August 15, 1902. The work of both seasons was pursued at the Marine Biological Laboratory, Woods Holl. In every instance the eggs for each experiment were taken from one female only. In this brief preliminary account detailed references to individual experiments are in some cases given, not to illustrate the condition peculiar to that experiment alone, but rather to set forth prevailing conditions.

Solutions.— These are rendered ineffective, (1) by the presence of foreign substances; (2) spermatozoa; (3) excessive number of eggs. The eggs of *Arbacia* are extremely sensitive to foreign substances liable to be introduced by the use of glassware not thoroughly cleansed or glassware previously used as receptacles

[1] Hunter, *American Journal of Physiology*, VI., 1901, p. 177.

for chemicals. For this reason it was found advisable to use only new glassware.

Much time was consumed in the work of sterilization to prevent contamination from spermatozoa. This, however, is essential. In the eighty-three experiments referred to sterilization was performed in accordance with the plan mentioned in the paper cited. If, after this treatment, there were eggs that developed through normal fertilization, such escaped notice. In five other experiments made to determine the necessity of this sterilization, the sea-water was not sterilized nor was the sea-urchin carefully washed in hydrant water. In these five experiments a few normally developing forms were noted.[1]

The relative proportion of eggs to concentrated sea-water is an important factor in determining the percentage of development. In the paper referred to mention was made of the necessity of placing comparatively few eggs in the solution. Tabulated results of some experiments on this point make the relative value of this condition more apparent. In two bowls containing equal amounts of condensed sea-water there were placed in the first bowl a greater number of eggs and in the second bowl very few. Out of the first bowl 14 per cent. reached the swimming gastrula stage; out of the second bowl 87.5 per cent. reached the same stage. Notes taken on early stages of the culture showed that in the bowl containing the less number, segmentation began in a greater number of eggs.

In other experiments an endeavor was made to measure approximately the number of eggs placed in a given amount of sea-water. One of these experiments is placed in tabulated form.

No. 1, 1 pipette full of eggs in 50 c.c. concentrated sea-water.

No. 2, 5 pipettes full of eggs in 50 c.c. concentrated sea-water.

No. 3, 10 pipettes full of eggs in 100 c.c. concentrated sea-water.

After two hours, transferred to sterilized sea-water, frequently changed at first — examined twenty-seven hours later with results as follows:

[1] The test of purity of culture was based on the facts, (1) that cleavage in parthenogenetically developing eggs of *Arbacia* at no time prior to blastula stage, resemble normal processes; (2) the shortest time in which any culture under constant observation reached the active swimming stage was nine hours and seven minutes, under like conditions normally fertilized embryos become active in about six hours; (3) the absence of the perivitelline membrane.

No. 1, 7 gastrulæ out of 16, 43 per cent.

No. 2, 9 gastrulæ out of 90, 10 per cent.

No. 3, 10 gastrulæ out of 109, 10 per cent.

Examined again twenty-six hours later with the following results :

No. 1, 8 plutei and 12 gastrulæ out of 27, 74 per cent.

No. 2, 1 pluteus out of 42, .02 per cent.

No. 3, no living forms out of 39.

This difference in the ratio of development is probably due to the noxious effects of the undeveloping eggs in the solution. This being the case a frequent change of the concentrated solution might raise the percentage, for while the sterilized sea-water was changed repeatedly the concentrated water was not changed at all during the period. The difference in results was evident at this stage, that is, the two lots of eggs when removed from the condensed solution showed a difference in behavior. As just noted, the eggs from the cultures containing the smaller number showed a larger percentage of segmentation. Cultures No. 2 and No. 3, having same ratios, gave similar percentages.

State of Development. — Wilson [1] has observed that the eggs of *Toxopneustes* which would not fertilize with spermatozoa gave some of the best results obtained with the magnesium solution. Delage [2] notes in *Strongylocentrotus* that frequently eggs which will not develop by artificial parthenogenesis are readily fertilized by spermatozoa. A number of observers have noted the wide variation in the behavior of the eggs of different females. Some eggs do not develop at all, others give large percentages of active larvæ. A case in point : An experiment with a large female, the eggs of which by their number, color and the freedom with which they came from the ovary — the ovaries in such cases when placed in sea-water lose form and become a mass of eggs — seemed to signify that the eggs were fully matured (oötids). As further proof, the greater part of these eggs were fertilized with spermatozoa, this resulted in the normal development of all the eggs observed. The remainder of the eggs were subjected to the influence

[1] E. B. Wilson, " A Cytological Study of Artificial Parthenogenesis in Sea-urchin eggs," *Archiv f. Entwickelungsmech.*, XII., 4, 1901, p. 535.

[2] Delage, Y., " Études Expérimentales sur la Maturation Cytoplasmique Chez les Echinodermes," *Archiv d. Zoöl. Exper. et Generale*, 3, Ser., IX., 1901, p. 300.

of condensed sea-water for two hours and four minutes, resulting in the appearance of many cytasters (Wilson) and the segmentation of many eggs, but no active larvæ. Segmentation and development ceased after four hours in the sterilized sea-water. The ovaries of another female were teased in sea-water and a small number of pale eggs were obtained. From the condition of the ovaries, the color and the number of eggs it was evident that the eggs were not mature (oöcytes). These were placed for the same length of time in concentrated sea-water and then transferred to normal sea-water. More than 90 per cent. of these eggs reached the active larval state. Eggs of females brought directly from the bed of the ocean gave better results than those kept in the laboratory aquarium for a time. The higher temperature of the sea-water in the laboratory probably hastened maturation. It became evident throughout the later series of experiments that oöcytes gave satisfactory results and oötids gave negative results. It seems probable, therefore, that concentrated sea-water is effective in producing development in *Arbacia* only when its influence is brought to bear upon the oöcyte.

The interesting question naturally arises concerning the exact stage at which the solution is effective. If this influence causes retention of the second polar body and its assumption of the rôle of the spermatozoön the subject is at once brought into direct relation with Boveri's[1] theory of natural parthenogenesis. In Delage's[2] observations on the influence of carbon dioxide on *Asterias* he gives results to show that the moment of susceptibility of the eggs lies between the time when the nuclear membrane of the germinal vesicle begins to dissolve and the beginning of the resting period of the egg nucleus; and that the immediate cause does not concern the polar bodies but rather the suspension of maturation for a given period. Upon resumption the polar karyokinesis is not confined to one region of the egg, but instead becomes general and includes the whole egg. Consideration of this phase is curtailed by Delage's[3] own statement

[1] Th. Boveri, "Zellstudien," I., 1887, p. 73.

[2] Y. Delage, "Nouvelles Recherches sur la Parthenogenese Experimentale chez Asterias glacialis," *Archiv de Zoöl. Exper.*, 1902, p. 217.

[3] Y. Delage, "Etudes Experimentales sur la Maturation cytoplasmique chez les Echinodermes," *Archiv de Zoöl. Exper.*, 3 Ser., IX., 1901, p. 295.

that phenomena manifested in the starfish must not be assumed to occur in the sea-urchin, and further according to the same author[1] the eggs of *Strongylocentrotus* are mature before being subjected to the solution. From this it would seem that there is a difference in the behavior of the eggs of *Arbacia* and *Strongylocentrotus*. In *Arbacia* it did not appear that in the development of the egg there was only one opportune moment when the concentrated solution was effective, but rather that ovarian eggs placed in the concentrated solution, were influenced to maturate and that maturation brought about in this way resulted, when the eggs were removed to normal sea-water, in segmentation and subsequent development. Experiments with eggs apparently mature frequently give small percentages, one to five per cent. of larval development. This may be accounted for by the presence of a few oöcytes in the ovaries. The difficulty in the case of *Arbacia* is that owing to the opacity of the eggs it is not possible to ascertain their exact state when placed in the concentrated solution.

There is some evidence which probably bears upon the question to be found in the examination of sections. Without entering into a detailed description at this time we find in iron-hæmatoxylin sections a heavily staining body in contact with the nuclear membrane. In some cases astral rays extend out from this dark body. In others these rays are absent. Later prophases occur, such as the elongation of the nucleus with an aster at each pole, followed by the mitotic figure in its various phases. In other words, there appear to be processes closely resembling normal karyokinesis. This conspicuous dark body shows its attitude towards the nucleus in cases where the dark body has failed to divide. In such cases, the nucleus is elongated on the side of contact and the chromatin is aggregated on the same side. It seems reasonable, therefore, to say that in these parthenogenetic eggs there is a force whose behavior approximates that of the spermatozoön.

Briefly, then, in the sea-urchin egg maturation takes place in the ovary before normal oviposition.[2]

[1] *Ibid.*, pp. 296, 301, 324.

[2] E. B. Wilson, "The Cell," 1900, p. 236.

The eggs used in these experiments were not deposited naturally, but were from ovaries removed from the female.

The ovaries thus taken were of two kinds: first, dark red in color, delicate in structure; when placed in sterilized sea-water eggs flowed freely from them without cutting or teasing; second, light red in color, firm in structure; comparatively few eggs obtained even after ovaries are cut and teased.

The eggs from ovaries of the first class gave unsatisfactory results when subjected to influence of concentrated sea-water, satisfactory results when fertilized with spermatozoa.

The eggs from ovaries of the second category have given percentages as high as 80 to 90, of parthenogenetic swimming forms, when subjected to influence of concentrated sea-water for the proper period.

Sections through ovaries, typical of this second class, reveal large numbers of oöcytes determined as such by the presence of the prominent germinal vesicle. Sections through thirty-two different follicles were examined. Only those showing germinal vesicle (oöcytes) or egg-nucleus (oötids) were counted. In these thirty-two sections of follicles there were 183 oöcytes and 85 oötids.[1] Oöcytes much smaller than normal eggs were not counted. The percentage of forms developed parthenogenetically is thus shown to bear a direct relation to the number of oöcytes in the culture.

It seems reasonable, then, to infer that the concentrated sea-water acts effectively upon the oöcyte only. The exact nature of this action it is hoped subsequent study will determine.

Duration.—The eggs of *Arbacia*, as is well known, are not sufficiently transparent to permit close observation upon the activity of the cell contents. For that reason I have been unable to note in the egg any definite appearance which would signify the proper moment for transference from condensed sea-water to sterilized sea-water. In a few cases I have found wide variations in the time that the eggs can be transferred and yet develop. The shortest time was one hour and twenty-two minutes. The limits within which the eggs from a given culture could be removed and yet develop were

[1] These follicles were from the ovaries of one female. Of the utilized eggs from this female fully 75 per cent. became swimming larvæ.

relatively narrow. It seems that the critical moment does not lie, as in the case of Delage's observations on *Asterias*, in the time when the eggs are placed in the solution, but rather when they are removed from the solution.

The stage of development of eggs when placed in solution evidently has some bearing on the time required for development. The differences in the states of ovarian eggs would seem to account for the differences in the time required for development, not only for the eggs of different individuals, but as in the experiment given below, for the eggs of the same individual.

The culture just referred to, the one in which larval development was obtained after an hour and twenty-two minutes in the concentrated solution, was one of a series of experiments to determine the proper length of time and also the time in which eggs of a given female will develop. This experiment also presented the longest period of time within which eggs of the same individual could be removed from the concentrated solution for subsequent development. The eggs were placed in the concentrated sea-water and allowed to remain one hour. Watch-glass cultures of approximately equal number of eggs, the standard being three pipette drops of eggs in each watch glass, were removed every two minutes from the concentrated solution and placed in sterilized sea-water. The length of time that eggs remained in the concentrated solution is given and opposite are the observations made, beginning seven hours and twenty minutes later.

Minutes in Concentrated Solution.	Notes Taken Seven to Ten Hours Afterward.
62.	No segmentation.
64.	A number of fragments from a few eggs that had segmented and then broken.
66.	The same.
68.	The same.
70.	Fragments more abundant but nothing in the nature of a cluster of blastomeres.
72.	The same.
74.	The same.
76.	Not so many fragments, a few eggs segmented into two and three blastomeres.

78. Very few whole eggs, nearly all in fragments of halves and less sizes.
80. Blastomeres remaining together, few fragments.
82. Three active well-formed blastulæ (examination made ten hours after removal from concentrated sea-water).
82–98. The eight cultures taken out during this time showed about the same percentage of development as 82.
100. No segmentation in this culture nor in any of the subsequent cultures.

This experiment shows a duration of sixteen minutes within which eggs were removed and larval development ensued. This was the widest range of the series. In many experiments a difference of five minutes on either side of the optimum moment determined the life of the culture. In all cases, as noted by other observers, eggs removed from the concentrated solution after a brief period begin to segment but do not continue to develop until they reach the swimming blastula stage. Eggs permitted to remain too long plasmolyze when placed in sterilized sea-water. As a result of this series of experiments the optimum period was determined at two hours. In each case three cultures were formed of the eggs, one of five minutes before the period, one at the period, and the other five minutes after the two hours.

Temperature. — The most favorable temperature obviously is the normal temperature of sea-water. Sudden changes caused by the use of water of a different temperature for replenishing cultures is detrimental. Greeley[1] has shown that blastulæ can be developed parthenogenetically in concentrated sea-water at a temperature of 2°, 11° and at the room temperature of 23°. I am convinced that uniform results cannot be obtained from cultures kept on the laboratory table. The changes in temperature which occur between day and night materially affect the behavior of the eggs. For this reason towards the close of the season the bowls containing the solutions were surrounded by running sea-water. This insures constancy of temperature as well as approximates the normal temperature.

[1] A. W. Greeley, Biol. Bulletin, IV., No. 3, p. 132.

Summary.

1. The conditions governing the production of artificial parthenogenesis in *Arbacia* by the use of sea-water concentrated by evaporation to a definite volume, are purity of solutions, stage in development of ovarian eggs, duration in concentrated solution, temperature of solutions.

2. The efficacy of solutions is subject to the presence of foreign substances, spermatozoa, relative number of eggs in a given amount of concentrated sea-water, and temperature. Foreign substances are excluded through extreme care in the preparation of solutions; spermatozoa are eliminated by raising normal sea-water to 70 degrees, by sterilizing all instruments in the flame, by washing thoroughly the body of the sea-urchin and the hands of the operator for three brief periods under stream from the hydrant. Results are most constant at normal temperature of sea-water. Development is obtained at room temperatures 22° to 24°. Variations in temperature of solutions materially affect the development of the culture.

3. The concentrated solution appears to be effective in producing development in oöcytes only. By reason of the opacity of the egg it is difficult to ascertain the exact stage or subsequent behavior in concentrated solution.

4. The average optimum period for eggs in concentrated solution lies between one hour and fifty-five minutes and two hours and five minutes.

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April 4, 1903.